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IL-2 withdrawal induces HTLV-1 expression through p38 activation in ATL cell lines

Miki Washiyama, Kazuo Nishigaki*, Nursarat Ahmed, Syuichi Kinpara, Yuichi Ishii, Noriyuki Kanzawa, Takao Masuda, Mari Kannagi

Department of Immunotherapeutics, Tokyo Medical and Dental University, Graduate School, Tokyo 113-8519, Japan

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Abstract **Expression of human T-cell leukemia virus type-1 (HTLV-1) in adult T-cell leukemia (ATL) cells is known to be marginal in vivo and inducible in short-term culture. In this study, we demonstrated that withdrawal of interleukin (IL)-2 from IL-2-dependent ATL cell lines resulted in induction of HTLV-1 mRNA and protein expression, and that viral induction was associated with phosphorylation of the stress kinase p38 and its downstream CREB. Pharmacological inhibitors of the p38 pathway suppressed viral expression induced by IL-2 depletion. These results indicate that the stress-induced p38 pathway might up-regulate HTLV-1 gene expression through at least CREB activation.**

Keywords: HTLV-1; p38; Expression; CREB; IL-2

1. Introduction

Human T-cell leukemia virus type-1 (HTLV-1) is a retrovirus, with a wide geographic distribution, which causes adult T-cell leukemia (ATL) [1,2] and neurological disorder, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. HTLV-1 antigens are hardly detectable in freshly isolated ATL cells from peripheral blood, but are spontaneously induced in the cells in 1-day culture [5]. The mechanism of reactivation of HTLV-1 expression in vitro is unclear. When transferred to in vitro culture, ATL cells often require IL-2, indicating that the IL-2 signal transduction pathway may associate with their proliferation and survival. In this study, we investigated the effect of IL-2 withdrawal on HTLV-1 expression in IL-2-dependent ATL cell lines, and unexpectedly found that IL-2 withdrawal resulted in the induction of HTLV-1 expression. We further elucidated the molecular mechanisms underlying this phenomenon, which involved activation of the p38 MAP kinase pathway.

2. Materials and methods

2.1. Cell line

ILT-Hod [6] and ILT-#92 [5] cells are IL-2-dependent HTLV-1-infected human T-lymphocytes derived from chronic ATL patients. These cell lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) with human IL-2 (10 U/ml) (Shionogi, Osaka, Japan) or human IL-15 (1 ng/ml) (Sigma).

2.2. Inhibitors

The p38 protein kinase inhibitors SB203580 and SB202190 were purchased from Calbiochem (La Jolla, CA, USA). The inhibitors were reconstituted in dimethyl sulfoxide (DMSO) to make a 1 mg/ml stock solution.

2.3. Western blot analysis

Cell lysates were prepared in lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and protease inhibitor cocktail (Calbiochem). Whole cell lysates were separated by electrophoresis on Tris/glycine minigels (Invitrogen) and then transferred electrophoretically to nitrocellulose filters for Western blotting with anti-phospho p38, anti-phospho Jun N-terminal kinase (JNK), anti-phospho MEK, anti-p38, anti-phospho cAMP responsive element binding protein (CREB) and anti-CREB antibodies (Cell Signaling) followed by visualization using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis was performed on scanned filters using ImageJ 1.37v software (http://rsb.info.nih.gov/ij/).

2.4. Flow cytometric analysis

For intracellular HTLV-1 staining, cells were permeabilized and fixed with 1% paraformaldehyde containing 20 μg/ml lysolecithin (Sigma) at room temperature for 2 min and 99% methanol on ice for 15 min, then incubated in 0.1% Triton-X in PBS for 5 min at 4 °C. The cells were incubated with mouse anti-HTLV-I Gag monoclonal antibody (mAb) (Gin-7) [7] and then incubated with FITC-conjugated goat anti-mouse IgG+M antibody. Ascites from BALB/c mice were used as a negative control for the first antibody. For intracellular phospho-CREB staining, the cells were permeabilized and fixed as above and incubated with the phospho-CREB (Ser 133) Rabbit mAb conjugated to Alexa Fluor 488 (Cell Signaling) and Rabbit isotype IgG conjugated to Alexa Fluor 488 (Cell Signaling) served as a negative control. For intracellular two-color staining for phospho-CREB and HTLV-I Gag, cells permeabilized and fixed as above were incubated with mouse anti-Gag mAb and stained with anti-mouse IgG conjugated to PE-Cy5 (Santa Cruz Biotechnology), and then stained with the phospho-CREB mAb conjugated to Alexa Fluor 488. Cells were analyzed using the FACScan flow cytometer. To detect apoptotic cells,

*Corresponding author. Present address: Department of Veterinary Medicine, Yamaguchi University, Yamaguchi 753-8515, Japan.
Fax: +81 3 83 933 5820.
E-mail address: kaz@yamaguchi-u.ac.jp (K. Nishigaki).

Abbreviations: ATL, adult T-cell leukemia; CREB, cAMP responsive element binding protein; Erk, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; HTLV-1, human T-cell leukemia virus-1; IL-2, interleukin 2; JNK, Jun N-terminal kinase; LTR, long-terminal repeat; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline

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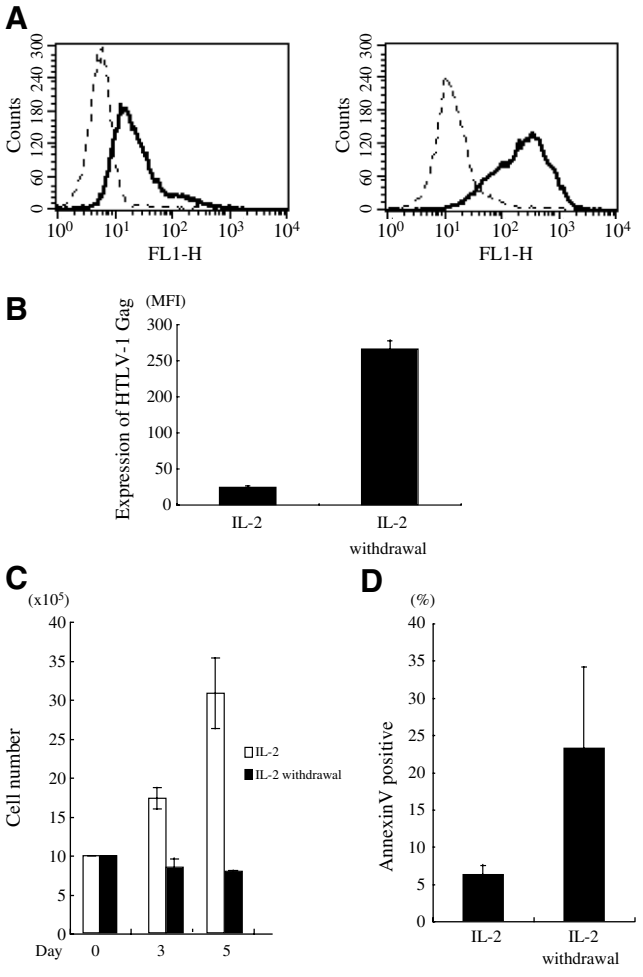

Fig. 1. Induction of HTLV-1 Gag expression following IL-2 withdrawal in ILT-Hod cells. (A) Intracellular HTLV-1 Gag proteins in ILT-Hod cells, which were cultured with (left panel) or without (right panel) IL-2 (10 U/ml) for 5 days, were stained with GIN-7 antibody (solid line) or control ascitis (dotted line) following permeabilization and fixation, and analyzed using a flow cytometer. Culture medium was replaced every 2 days. One representative result is shown. (B) MFI of intracellular HTLV-1 Gag expression from three sets of experiments, performed as shown in (A), is shown, along with the S.D. ($P < 0.05$). (C) Live cells of ILT-Hod cultures in the presence (open bar) or absence (closed bar) of IL-2 (10 U/ml) were counted at 3 and 5 days by a trypan blue exclusion method. (D) Apoptotic cells in ILT-Hod cultures in the presence or absence of IL-2 for 5 days were stained with annexinV-PE, and the ratio was analyzed by flow cytometry. Graphs represent the means ± S.D. from triplicate samples.

1×10^5 cells were washed with PBS and stained with annexinV-PE (BD Pharmingen Co.) followed by flow cytometric analysis.

2.5. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (0.5 μg) extracted from cells by using Isogen (Nippon Gene) were treated with DNase (Ambion) and subjected to RT-PCR with HTLV-I *gag*-specific primers (5′-CCTTACCACGCCTTCGTAGAACGCCTCAACATAGC-3′ for forward, 5′-TTTGTCTTTGGGGGTCCAGGTCTGACAAGCCCGCA-3′ for reverse) by using Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) following reverse transcription with oligo-dT primers. PCR cycles consist of an initial denaturation step at 95 °C for 5 min and 40 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 10 s, extension at 72 °C for 10 s, and denaturation at 85 °C for 2 s. RT-PCR products were quantified and standardized by simultaneously quantified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA copy number.

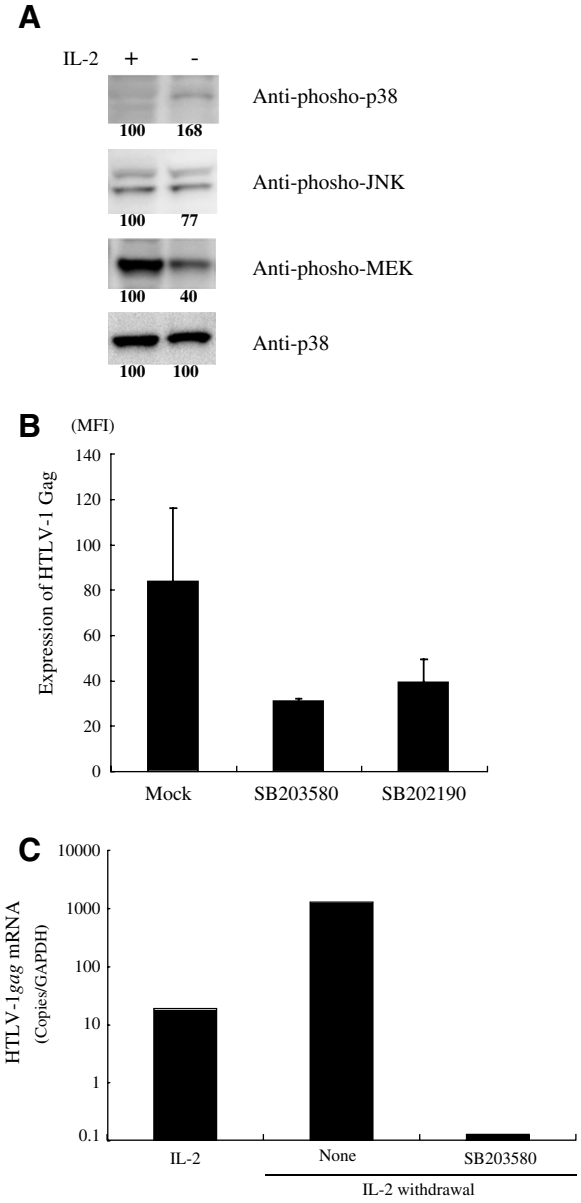

Fig. 2. Association of p38 activation with HTLV-1 Gag induction in ILT-Hod cells following IL-2-depletion. (A) ILT-Hod cells were cultured in the presence (+) or absence (−) of IL-2 for 5 days, and Western blot analysis of total cell lysates was carried out using anti-phospho-p38, anti-phospho-JNK, anti-phosho-MEK and anti-p38 antibodies. The values at the bottom end of each lane represent relative densities against those of IL-2 presence (+). (B) Intracellular HTLV-1 Gag expression in ILT-Hod cells cultured with SB203580 (10 μM), SB202190 (10 μM) or DMSO control (Mock) in the absence of IL-2 for 5 days. The culture medium was replaced with fresh drugs or DMSO at day 3. Graphs represent the mean ± S.D. from triplicate samples. (C) HTLV-I *gag* mRNA expression in ILT-Hod cells that were cultured for 24 h under indicated conditions. Values indicate relative *gag*-mRNA copy numbers against those of GAPDH.

3. Results and discussion

3.1. Induction of HTLV-1 antigens by IL-2 withdrawal in IL-2-dependent ILT-Hod cells

ILT-Hod cells, an IL-2 dependent T-cell line established from peripheral blood mononuclear cells from a patient with chronic ATL, express low levels of HTLV-1 antigens, includ-

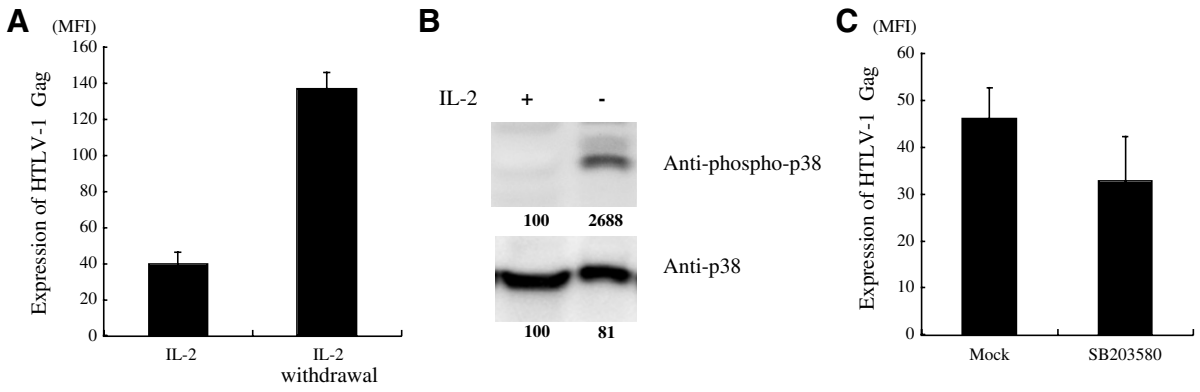

Fig. 3. Induction of Gag expression and p38 phosphorylation in ILT-#92 cells by IL-2 depletion. (A) Intracellular HTLV-1 Gag expression in ILT-#92 cells cultured with or without IL-2 (10 U/ml) for 5 days was analyzed by flow cytometry, and MFI from three samples is shown, along with the S.D. ($P < 0.05$). (B) Western blot analysis using anti-phospho-p38 antibody (top) of total cell lysates from ILT-#92 cells cultured in the presence or absence of IL-2 for 5 days. The filter was stripped and then incubated with anti-p38 antibody to determine total p38 kinase level (bottom). The values at the bottom end of each lane represent relative densities against those of IL-2 presence (+). (C) Intracellular HTLV-1 Gag expression in ILT-#92 cells with SB203580 (10 μM) or DMSO control (Mock) in the absence of IL-2 for 5 days. The culture medium was replaced with fresh drugs or DMSO at day 3. Graphs represent MFI ± S.D. from triplicate samples.

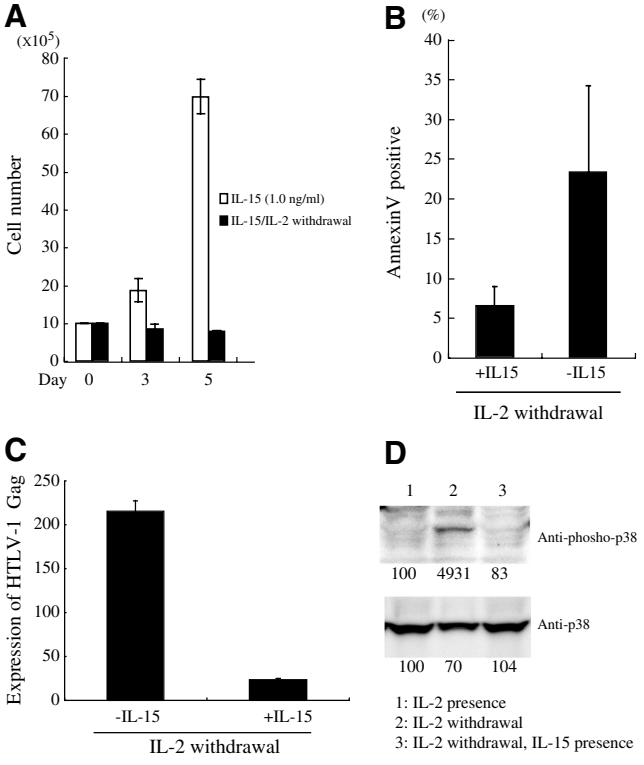

Fig. 4. Effect of IL-15 on HTLV-1 Gag induction by withdrawal of IL-2 in ILT-Hod cells. (A) Live cell numbers in ILT-Hod cultures in the presence (open bar) or absence (closed bar) of IL-15 (1 ng/ml) were counted by a trypan blue exclusion method 3 and 5 days after withdrawal of IL-2. (B) Apoptotic cells in ILT-Hod cultures in the presence or absence of IL-15 for 5 days after IL-2 withdrawal were stained with annexin V and analyzed by flow cytometry. Graph represents mean ratio ± S.D. from triplicate samples. (C) ILT-Hod cells maintained in the presence of IL-2 (10 U/ml) were washed and further cultured with or without IL-15 (1.0 ng/ml) in the absence of IL-2 for 5 days, and intracellular HTLV-1 Gag expression was measured. MFI ± S.D. from triplicate samples is shown ($P < 0.05$). (D) Western blot analysis of total cell lysates from ILT-Hod cells treated as indicated was performed using anti-phosphor-p38 antibody (top). The filter was stripped and then incubated with anti-p38 antibody to determine total kinase level (bottom). The values at the bottom end of each lane represent relative densities against those of IL-2 presence (+).

ing Gag and Tax proteins [5]. To examine if HTLV-1 gene expression is regulated by IL-2, we compared the expression of HTLV-1 Gag in ILT-Hod cells cultured in the presence or absence of IL-2 for 5 days. As shown in Fig. 1A, intracellular HTLV-1 Gag expression in ILT-Hod cells was significantly increased following withdrawal of IL-2. The mean fluorescence intensity (MFI) of Gag expression in the absence and presence of IL-2 was 215.6 and 23.9, respectively ($P < 0.05$) (Fig. 1B).

The number of ILT-Hod cells increased more than three times in culture with IL-2 for 5 days, while it slightly decreased without IL-2 (Fig. 1C). IL-2 withdrawal resulted in an increase in the number of apoptotic cells (23.2%, $P = 0.054$), as determined by annexin V staining (Fig. 1D). Fujimura et al. have reported that IL-2 withdrawal in ATL cell lines causes arrest in the G1 phase and subsequent apoptosis [8]. However, enhancement of Gag expression was not restricted to the G1 fraction of ILT-Hod cells following IL-2-depletion (data not shown), indicating that HTLV-1 induction was not associated with a certain stage of the cell cycle.

3.2. p38α regulates HTLV-1 Gag expression

It has been shown that withdrawal of cytokines results in activation of stress kinases, p38 and JNK in IL-7- or IL-3-dependent lymphoid cells [9,10]. To determine if stress-induced mitogen-activated protein kinases (MAPKs) such as p38 and JNK are affected in ILT-Hod cells by withdrawal of IL-2, we carried out Western blot analysis using phospho-p38 and phospho-JNK antibodies. As shown in Figs. 2A and 4D, phosphorylation of p38 was induced in ILT-Hod cells following IL-2 withdrawal for 5 days. In contrast, JNK was constitutively phosphorylated in ILT-Hod cultures with or without IL-2. MEK, a kinase upstream of extracellular signal-regulated protein kinase, was also phosphorylated in ILT-Hod cells cultured in the presence of IL-2, and IL-2 withdrawal resulted in a decrease in MEK phosphorylation probably due to decreased signal from IL-2 receptor (Fig. 2A). In fact, MEK phosphorylation was induced by IL-2 stimulation in ILT-Hod cells (data not shown). The total amount of p38 did not change following withdrawal of IL-2 for 5 days. These results indicate that stress-induced p38 activation correlates with the induction of

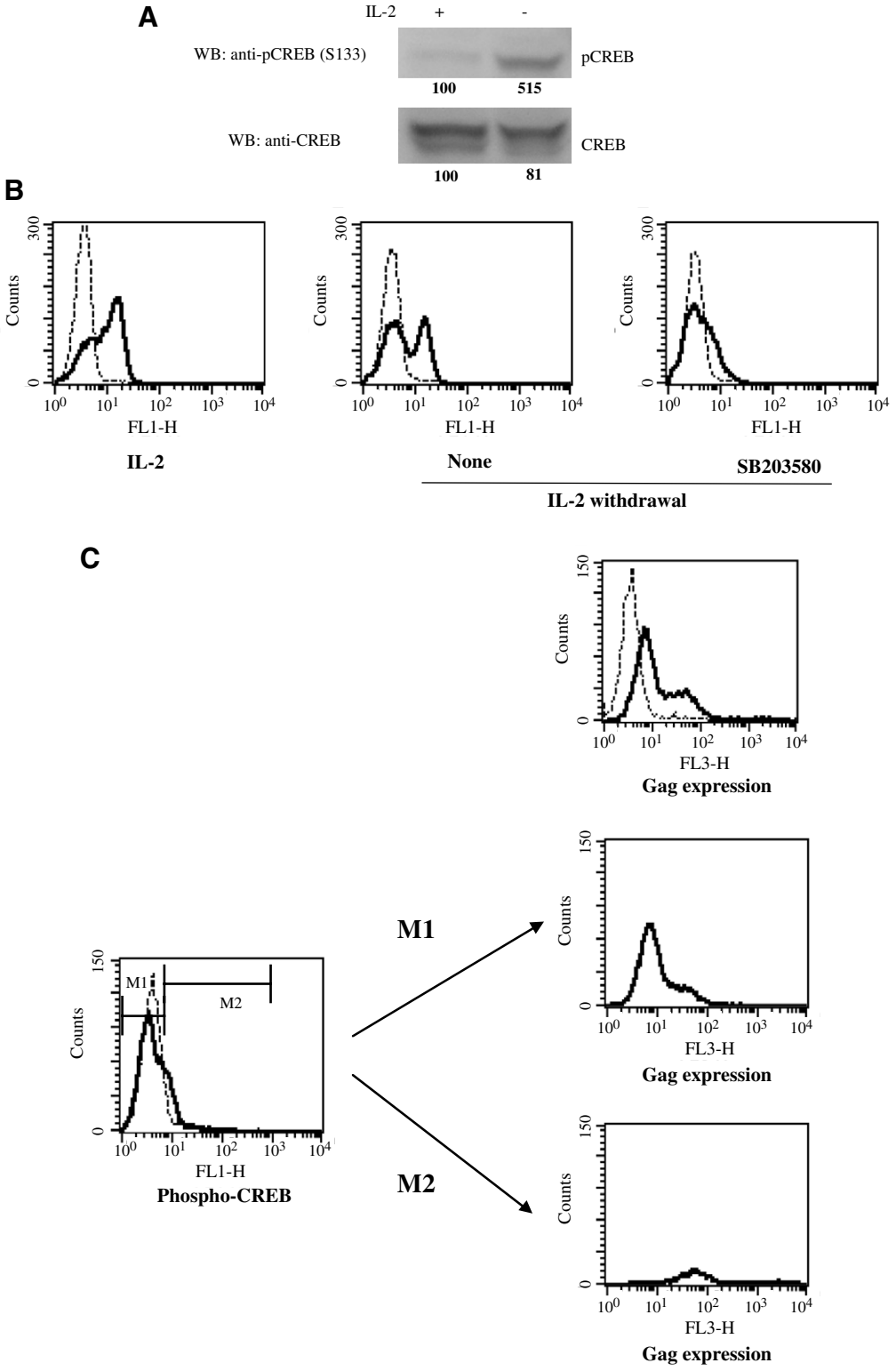

Fig. 5. Phosphorylation of CREB by withdrawal of IL-2 in ILT-Hod cells (A). ILT-Hod cells were cultured in the presence or absence of IL-2 for 5 days, and Western blot analysis of total cell lysates was carried out using anti-phospho-CREB (top) and anti-CREB (bottom) antibodies. The values at the bottom end of each lane represent relative densities against those of IL-2 presence(+). Suppression of CREB-phosphorylation by p38-inhibitor (B). ILT-Hod cells were cultured in the absence of IL-2, with or without SB203580 (10 μM) as indicated for 3 days, and intracellularly stained with Alexa Fluor 488-conjugated rabbit anti-posphorylated CREB antibody. Dotted line indicates the staining with control antibody. Preferential HTLV-I-expression in cells with activated CREB (C). ILT-Hod cells were cultured without IL-2 for 5 days, and intracellularly stained with mouse anti-Gag mAb followed by staining with PE-Cy5-conjugated anti-mouse antibody and then stained with Alexa Fluor 488-conjugated anti-phospho-CREB Rabbit mAb or isotype Rabbit IgG. According to the amounts of phosphorylated CREB in the cells (left), cells were divided to two populations without (M1) or with (M2) phosphroylated CREB, and HTLV-I Gag expression was analyzed in the whole cell (right top), M1 (right middle), and M2 (right bottom) populations. Dotted lines indicate staining with control rabbit IgG (left) or mouse ascites (right).

HTLV-1 antigen in IL-2-dependent ILT-Hod cells. We further examined the effects of p38 inhibitors on HTLV-1 expression, and found that treatment with either SB203580 or SB202190 significantly inhibited HTLV-1 Gag induction in ILT-Hod cells both at a protein level (Fig. 2B) and a mRNA level (Fig. 2C) following withdrawal of IL-2 for 5 days or 24 h, respectively.

Similar results were obtained in ILT-#92 cells, another IL-2-dependent T-cell line established from an ATL patient (Fig. 3). Intracellular Gag expression in ILT-#92 cells increased from 39.8 to 136.7 (MFI) as determined by flow cytometry, following IL-2 withdrawal for 5 days ($P < 0.05$) (Fig. 3A). The phosphorylated p38 in ILT-#92 cells that was not detectable in the presence of IL-2 was induced by withdrawal of IL-2 for 5 days (Fig. 3B). Similar induction of p38 phosphorylation following IL-2 withdrawal was observed in two other IL-2 dependent HTLV-I-infected cell lines derived from ATL patients (data not shown). Treatment of ILT-#92 cells with p38 inhibitor SB203580 partly inhibited HTLV-1 Gag induction following withdrawal of IL-2 ($P = 0.0315$) (Fig. 3C). These results indicate that the activation of p38 was, at least in part, responsible for the induction of HTLV-1 gene expression in this system.

3.3. IL-15 inhibits the expression of HTLV Gag induced by IL-2 withdrawal

IL-15 is constitutively produced by various non-hematopoietic cells in vivo, and is also known to support growth of HTLV-1-infected cells in vitro as well as IL-2 [11]. We examined the effect of IL-15 on HTLV-1 expression and p38 phosphorylation induced by withdrawal of IL-2. ILT-Hod cells cultured with IL-15 expanded approximately seven times in 5 days (Fig. 4A), and contained a smaller number of apoptotic cells than in culture without IL-2 or IL-15 (Fig. 4B). Induction of intracellular expression of HTLV-1 Gag in ILT-Hod cells by IL-2 withdrawal was significantly inhibited in the presence of IL-15 (Fig. 4C) ($P < 0.05$). As shown in Fig. 4D, the induction of p38 phosphorylation by IL-2 withdrawal was inhibited in the presence of IL-15, to the basal level. Thus, IL-15 can substitute for IL-2 in ILT-Hod cells.

3.4. Involvement of CREB activation in HTLV-I expression by IL-2 withdrawal in ILT-Hod cells

Finally, we determined if CREB was activated in ILT-Hod cells following IL-2 depletion, since CREB is located downstream of p38α and is also known to be a critical transcription factor for HTLV-1-expression [12]. Western blot analysis using anti-phosphor-CREB antibody that recognizes the phosphorylated CREB at Ser133, revealed that the phosphorylation of CREB was induced by IL-2 withdrawal, although a very low level of phosphorylated CREB was detected in the presence of IL-2 (Fig. 5A). The total level of CREB was equivalent in ILT-Hod cells either in the presence or absence of IL-2 (Fig. 5A). Intracellular staining of phosphorylated CREB and its FACS analysis revealed that activation of CREB following IL-2 withdrawal was inhibited by SB203580 in ILT-Hod cells (Fig. 5B). Unlike the result from Western blot analysis, the levels of CREB phosphorylation did not dramatically change by IL-2 withdrawal in this setting but exhibited two peaks (Fig. 5B). Further analysis on the two cell populations indicated that phosphorylated CREB was preferentially detected in cells that highly expressed HTLV-I (Fig. 5C). These data combined with above suggested that activation of CREB appeared to be associated with viral induction following IL-2 withdrawal.

In the present study, survival and growth of ATL cell lines depend on the IL-2/IL-15 signal transduction pathway. In these cells, p38 was not activated unless the cellular stress signaling was turned on by withdrawal of IL-2/IL-15. Stress-induced activation of the p38 MAPK pathway resulted in up-regulation of HTLV-1 expression, at least through CREB-mediated transcription. These findings suggest a role for IL-2/IL-15 signaling pathways in HTLV-1 gene silencing in vivo, and involvement of the p38 MAPK pathway in HTLV-1 induction, which may be modified by inflammation, cell growth, cell differentiation, cell cycling and cell death [13].

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